LETTER

doi:10.1038/nature09908

CPEB and two poly(A) polymerases control miR-122 stability and p53 mRNA translation

David M. Burns[1], Andrea D'Ambrogio[1], Stephanie Nottrott[1] & Joel D. Richter[1]

Cytoplasmic polyadenylation-induced translation controls germ cell development[1,2], neuronal synaptic plasticity[3–5] and cellular senescence[6,7], a tumour-suppressor mechanism that limits the replicative lifespan of cells[8,9]. The cytoplasmic polyadenylation element binding protein (CPEB) promotes polyadenylation by nucleating a group of factors including defective in germline development 2 (Gld2), a non-canonical poly(A) polymerase[10–12], on specific messenger RNA (mRNA) 3′ untranslated regions (UTRs). Because CPEB regulation of *p53* mRNA polyadenylation/translation is necessary for cellular senescence in primary human diploid fibroblasts[6], we surmised that Gld2 would be the enzyme responsible for poly(A) addition. Here we show that depletion of Gld2 surprisingly promotes rather than inhibits *p53* mRNA polyadenylation/translation, induces premature senescence and enhances the stability of *CPEB* mRNA. The *CPEB* 3′ UTR contains two miR-122 binding sites, which when deleted, elevate mRNA translation, as does an antagomir of miR-122. Although miR-122 is thought to be liver specific, it is present in primary fibroblasts and destabilized by Gld2 depletion. Gld4, a second non-canonical poly(A) polymerase, was found to regulate *p53* mRNA polyadenylation/translation in a CPEB-dependent manner. Thus, translational regulation of *p53* mRNA and cellular senescence is coordinated by Gld2/miR-122/CPEB/Gld4.

Mouse embryo fibroblasts (MEFs) derived from *CPEB* knockout mice do not senesce as do MEFs derived from wild-type mice, but instead are immortal. Senescence is rescued when ectopic CPEB is expressed in the knockout MEFs and potentiated when expressed in wild-type MEFs[7]. Human foreskin fibroblasts depleted of CPEB also bypass senescence and divide for approximately 270 days compared with wild-type cells, which senesce after about 90 days. As with the mouse cells, ectopic expression of CPEB rescues senescence in knockdown cells and potentiates senescence in wild-type cells. CPEB controls the polyadenylation-induced translation of *p53* mRNA, and indeed CPEB-induced senescence requires p53. Depletion of CPEB also induces the 'Warburg effect', where mitochondrial respiration is reduced and cells produce ATP primarily through glycolysis[6].

To investigate the possibility that CPEB control of *p53* polyadenylation requires Gld2, human primary foreskin fibroblasts were stably transduced with lentiviruses expressing two different short hairpin RNA (shRNAs) against the Gld2 coding sequence. Surprisingly, Gld2 depletion (Fig. 1a, b) induced an increase in both p53 protein levels (Fig. 1c) and *p53* mRNA polyadenylation (Fig. 1d and Supplementary Fig. 1). Also unexpectedly, depletion of Gld2 resulted in increased oxygen consumption (Fig. 1e) and entry into a senescence-like cell-cycle arrest as evidenced by β-galactosidase staining at acidic pH (Fig. 1f). In comparison, CPEB-depleted cells had decreased oxygen consumption, fewer cells staining with β-galactosidase, increased lifespan and, most importantly, reduced poly(A) tail size on *p53* mRNA and approximately 50% reduction in p53 protein levels[6].

These paradoxical results prompted us to examine CPEB levels in Gld2-deficient cells because CPEB is required for normal *p53* mRNA translation[6]. After comparing the amounts of CPEB nuclear pre-mRNA by reverse transcription followed by quantitative PCR (RT–qPCR) and mostly cytoplasmic mRNA by exon-specific RT–qPCR, we found that the pre-mRNA levels, which generally reflect transcription, were nearly unchanged whereas cytoplasmic mRNA levels increased by about five-fold (Fig. 2a). Thus, in the absence of Gld2, *CPEB* mRNA unexpectedly was more stable.

Surmising that Gld2 might control p53 protein levels through CPEB, we next used a *Renilla* luciferase (Rluc) and firefly luciferase (Fluc) reporter system to investigate post-transcriptional regulation of CPEB by Gld2. As shown in Fig. 2b, c, the entire CPEB 3′ UTR was

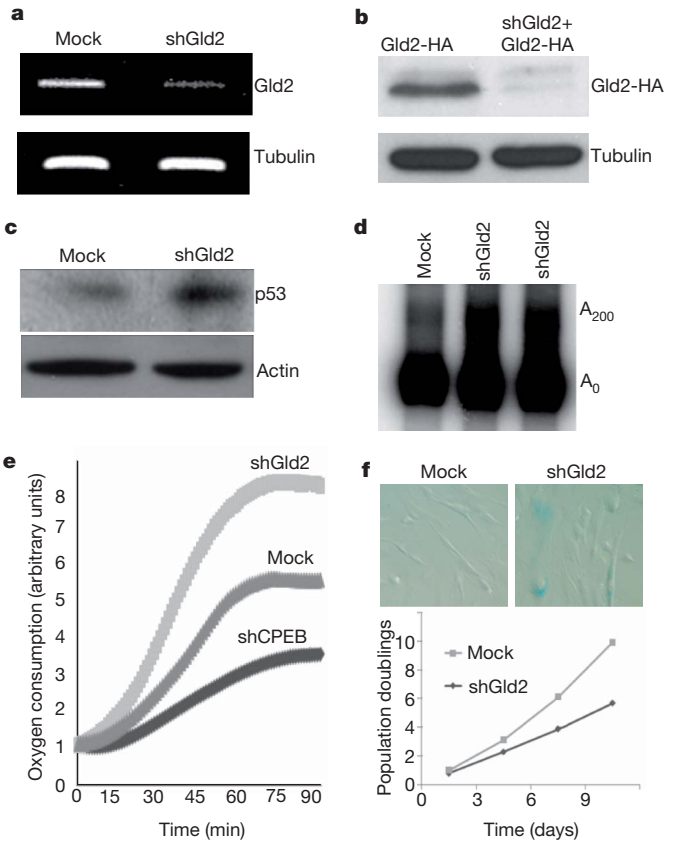

Figure 1 | Depletion of Gld2 enhances *p53* expression. **a**, RT–PCR of Gld2 and tubulin RNAs after infection of human foreskin fibroblasts with lentiviruses expressing shRNA against Gld2 or GFP (Mock, same in all panels). **b**, Knockdown of Gld2–HA in cells expressing ectopic Gld2–HA. Tubulin served as a loading control. **c**, Western blot showing 2.5-fold enhanced expression of p53 relative to tubulin after Gld2 depletion. **d**, Poly(A) tail analysis of *p53* mRNA in wild-type and Gld2-depleted cells (two shRNAs targeting different regions of Gld2 were used). **e**, Oxygen consumption in cells infected with shCPEB, shGld2, or empty vector (Mock). **f**, Mock or shGld2-infected cells stained for β-galactosidase, which denotes cellular senescence. Population doublings were determined in wild-type or Gld2-depleted cells.

[1]Program in Molecular Medicine, University of Massachusetts Medical School, Worcester, Massachusetts 01605, USA.

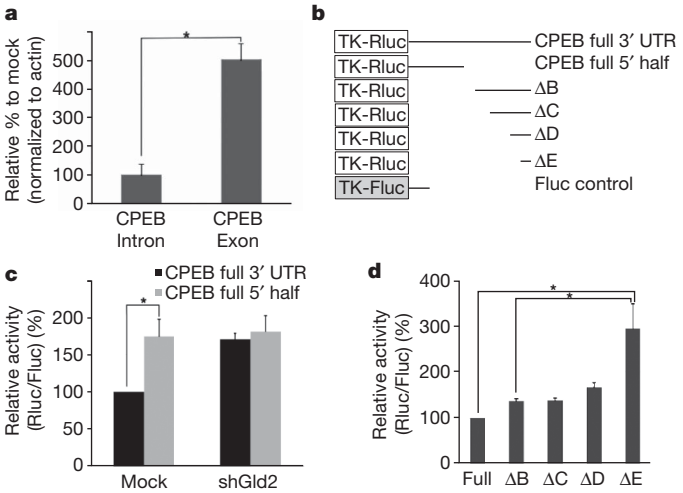

Figure 2 | Gld2 knockdown increases CPEB reporter mRNA and translation by a post-transcriptional mechanism. **a**, Fold change of nuclear (intron-containing) or predominantly cytoplasmic (exon-containing) CPEB RNA after Gld2 depletion ($n = 3$; bars, s.e.m.) **b**, Reporter constructs used in the following experiments (numbers refer to nucleotides of CPEB 3′ UTR). **c**, **d**, Cells expressing firefly luciferase (Fluc) as a control and *Renilla* luciferase (Rluc) as noted in **b** were depleted of Gld2; the amount of *Renilla* luciferase activity (relative to firefly) was derived from RNA containing the entire CPEB 3′ UTR (full) and set at 100. In all panels, $n = 3$; bars, s.e.m.; $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test).

translated about 40% less efficiently compared with a reporter lacking the 3′ most 455 nucleotides (mock). However, in Gld2-deficient cells, the two reporters were translated equally. Additional deletions (Δ) of the CPEB 3′ UTR suggested that there might be multiple regions that elicited increases in reporter translation after Gld2 knockdown (that is, ΔE translation was about twofold greater than ΔB, ΔC or ΔD translation) (Fig. 2d).

Analysis of the regions of the CPEB 3′ UTR that mediated translational repression by Gld2 revealed the presence of two potential miR-122 binding sites (Supplementary Fig. 2). Although miR-122 is thought to be liver-specific and account for approximately 70% of the total population of microRNAs in that tissue[13], deletion of these specific sites, either individually or combined, alleviated translational repression in Gld2-depleted cells (Fig. 3a), which were nearly identical to those observed with the large deletions (Fig. 2d). These results suggest that miR-122 might repress CPEB mRNA translation in human skin fibroblasts and indicate that this microRNA (miRNA) is more widely distributed than originally thought. Indeed, recent evidence shows that miR-122 is present in human skin[14] and even HEK293 cells[15].

To assess directly whether miR-122 might repress *CPEB* mRNA expression, we first cloned and sequenced it from human foreskin fibroblasts and found that it contained a non-templated 3′ monoadenylate residue (Fig. 3b; see discussion). Next, cells were electroporated with a locked nucleic acid (LNA) antagomir for miR-122, or as a control, a scrambled LNA. The miR-122 antagomir enhanced reporter expression by about 3.25-fold relative to control (Fig. 3c), but had no stimulatory effect on a reporter whose 3′ UTR contained no miR-122 sites (Supplementary Fig. 3). Based on evidence from Katoh *et al.*[16], who demonstrated that, in murine liver, Gld2 is essential for miR-122 stability, we suspected that Gld2 might influence CPEB expression and possibly *p53* mRNA translation by controlling miR-122 steady-state levels. Indeed, Fig. 3d demonstrates that depletion of Gld2 from skin fibroblasts reduced the level of miR-122 by nearly 40-fold. Importantly, when miR-122 LNA antagomir-transduced cells were incubated with the proteasome inhibitor MG132 and pulsed-labelled with [^{35}S]methionine for 15 min followed by p53 immunoprecipitation, there was a twofold increase in the synthesis rate of p53 (Fig. 3e, f). Taken together, these data demonstrate that human primary skin

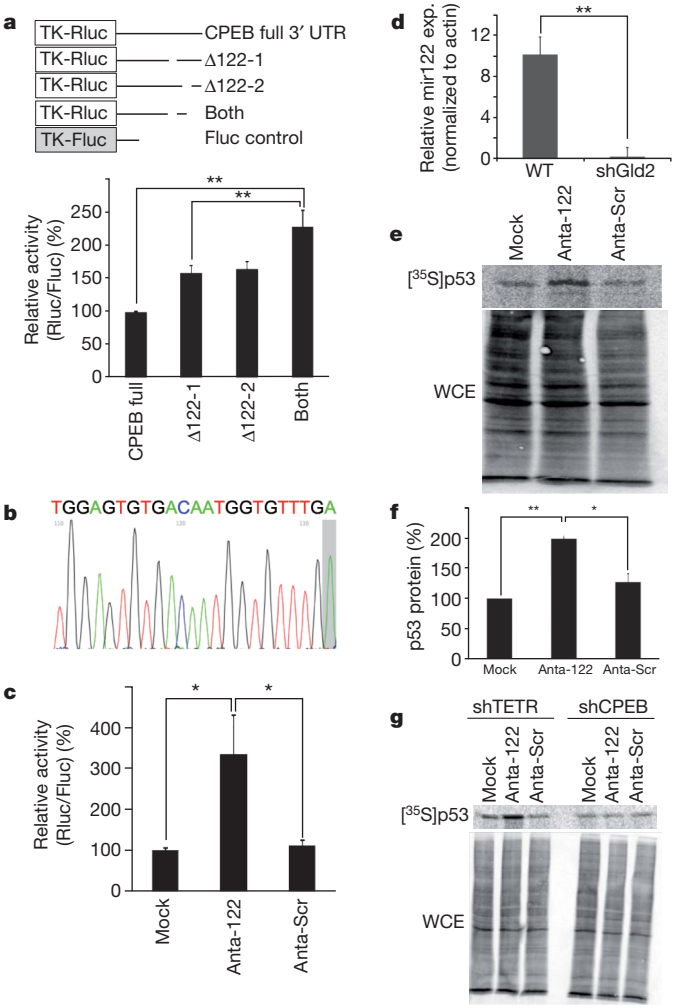

Figure 3 | miR-122 activates *p53* mRNA translation by repressing CPEB. **a**, Gld2-depleted fibroblasts were transduced with firefly and *Renilla* luciferase with CPEB full-length or deletion mutant 3′ UTRs lacking putative miR-122 sites (Supplementary Fig. 2). The data are expressed as in Fig. 2; in all panels, $n = 3$; bars, s.e.m.; $^{*}P < 0.05$, $^{**}P < 0.01$ (Student's *t*-test). **b**, Sequence of miR-122 from fibroblasts; a non-templated adenosine is shaded. **c**, Fibroblasts expressing firefly and *Renilla* luciferase containing the CPEB 3′ UTR were electroporated with miR-122 (Anta-122), scrambled (Anta-Scr) or no LNA antagomir (Mock); data are expressed as in Fig. 2. **d**, Quantitative RT–PCR for miR-122 in cells expressing GFP (wild type (WT)) or shGld2. **e**, **f**, Immunoprecipitation of [^{35}S]methionine-labelled p53 from MG132-treated cells transduced with no (mock–GFP), miR-122 (Anta-122) or scrambled (Anta-Scr) LNA antagomirs. WCE, whole cell lysate. **g**, Fibroblasts were treated as in **e**–**g** after first expressing either TET repressor (shTETR, a control) or *CPEB* shRNA.

fibroblasts contain miR-122 and that Gld2 controls its steady state levels or activity.

Although consistent with the hypothesis that miR-122 mediates *p53* mRNA translation through CPEB, these data do not eliminate the possibility that miR-122 could act through another molecule to regulate p53 synthesis (note that *p53* mRNA has no miR-122 sites according to Targetscan.org or Microrna.org). Consequently, we infected cells with a lentivirus expressing shRNA for CPEB as well as the miR-122 antagomir followed by a 15 min pulse of [^{35}S]methionine and p53 immunoprecipitation. Figure 3g shows that although miR-122 antagomir alone elicited an increase in p53 synthesis, the antagomir plus shRNA for CPEB induced no increase. Taken together, these data demonstrate that Gld2 activity stabilizes miR-122, which in turn reduces CPEB expression; CPEB then acts directly on *p53* mRNA to control poly(A) tail length and translation.

If not Gld2, what poly(A) polymerase modifies *p53* mRNA polyadenylation and translation? We surmised that an alternative non-canonical poly(A) polymerase, that is, one that lacks an RNA binding domain and thus would require another factor such as CPEB to be tethered to the RNA, would most probably be involved. Two cytoplasmic enzymes have this characteristic: Gld4 (PAPD5)[17] and MitoPAP (PAPD1)[18]. Both polymerases were depleted with shRNAs (Supplementary Fig. 4) but only the loss of Gld4 reduced p53 protein levels (Fig. 4a). To investigate whether Gld4 interacts with *p53* mRNA in a CPEB-dependent manner, Flag–Gld4 was expressed in cells (Supplementary Fig. 5) containing shRNA for tetracycline repressor (TETR, a control) or CPEB. Gld4 was then immunoprecipitated and the extracted RNA was examined for p53 and GAPDH (a control) RNAs by RT–PCR (Fig. 4b). *p53* mRNA was detected only when CPEB was present, suggesting that Gld4 is anchored to *p53* mRNA by CPEB, and indeed, CPEB co-immunoprecipitated Gld4 but not Mcl1, a non-specific control (Fig. 4c). Finally, depletion of Gld4 reduced *p53* mRNA polyadenylation (Fig. 4d), which probably then induced p53 mRNA destabilization (Fig. 4e; depletion of Gld4 reduced mostly cytoplasmic *p53* mRNA as examined by RT–PCR using exon-specific primers but had no effect on *p53* transcription as examined by RT–PCR using intron-specific primers).

The results presented here and in Katoh *et al.*[16] suggest a model for homeostatic control of p53 synthesis in human skin fibroblasts (Fig. 4f). Gld2 stabilizes miR-122 by catalysing the addition of a single adenylate residue to its 3′ end[16]. miR-122 then base-pairs to two regions of the CPEB 3′ UTR, causing instability and/or translational inhibition of the mRNA. CPEB, whose levels are modulated by these events, binds to the *p53* 3′ UTR and recruits Gld4, which in turn maintains *p53* mRNA polyadenylation and translation. We visualize this hierarchical regulation of p53 to resemble a rheostat, where translation is turned up or down rather than a switch, where translation is turned on or off[19], although *p53* mRNA translation can also be controlled by a switch mechanism in response to DNA damage[20,21]. A 50% change in p53 synthesis can toggle a cell between growth and senescence[6], demonstrating that drastic biological consequences result from a relatively modest change in protein level.

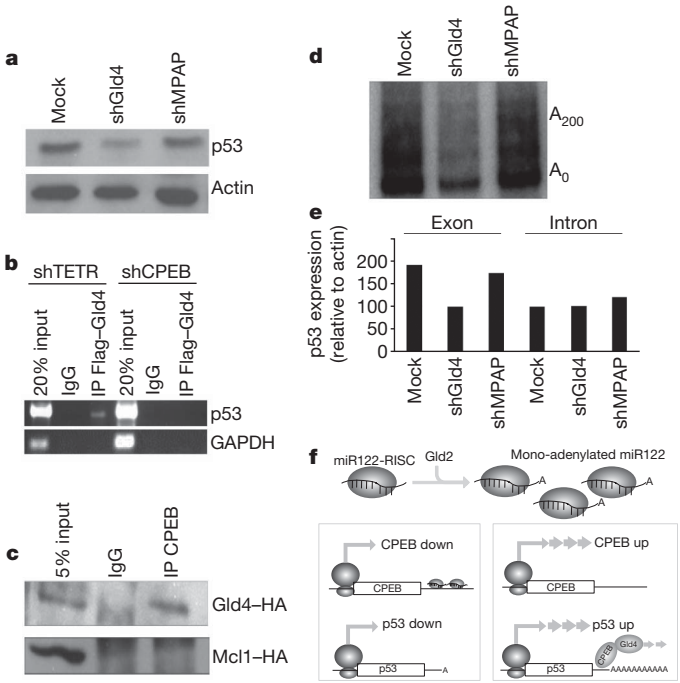

Figure 4 | Gld4 controls *p53* mRNA expression. **a**, p53 and actin western blots from fibroblasts expressing GFP (Mock), shGld4 or shMitoPAP (shMPAP). **b**, Fibroblasts containing *shTETR* or *CPEB* were transfected with Gld4–Flag followed by Flag antibody or IgG immunoprecipitation of RNA complexes and RT–PCR for *p53* or GAPDH (control) RNAs. **c**, Protein from fibroblasts infected with Gld4–HA and CPEB–Flag was Flag or IgG immunoprecipitated and western blotted for HA. Other cells infected with CPEB–Flag and MCl1–HA (a non-specific control) were processed similarly. **d**, Examination of *p53* poly(A) tail[6] from skin fibroblasts expressing GFP or (Mock) Gld4 or mitoPAP shRNAs. **e**, RT–PCR analysis of predominantly cytoplasmic *p53* RNA (exon-specific primers), or nuclear *p53* pre-mRNA (intron-specific primers) in cells expressing GFP (Mock) or Gld4 or MitoPAP shRNAs. **f**, Model for regulation of *p53* translation; see text for explanation.

Although ectopically expressed Gld2 immunoprecipitated from hepatocarcinoma cells adds a single adenosine to miR-122 *in vitro*[16], Gld2 tethered to a small non-coding RNA by MS2 adds more than 300 adenylate residues in injected oocytes[22], and about that same amount to mRNA when bound to CPEB[10]. How the enzyme can modulate its catalytic activity depending on the substrate is unknown, but we postulate that components of the RNA-induced silencing complex might be responsible. In addition to our demonstration that miR-122 is 3′ mono-adenylated in skin fibroblasts, approximately 20% of all RNA deep sequencing reads from cloned neuroblastoma miRNAs contain a non-templated adenylate residue[23], suggesting that miR-122 may be one of several miRNAs that are mono-adenylated by Gld2.

In conclusion, our results demonstrate that Gld2 control of miR-122 stability in human skin fibroblasts tunes CPEB expression, which in turn regulates *p53* mRNA polyadenylation and translation by Gld4. The coordinated activities of these factors then gate entry into senescence. These studies also bring up two additional aspects of CPEB-related activities: how does Gld4, but not Gld2, associate with CPEB on *p53* mRNA, and what molecular machinery is responsible for miR-122 destruction upon Gld2 depletion? Deciphering the mechanisms involved would probably require analysis of the combinatorial associations of factors on different RNA substrates.

METHODS SUMMARY

Molecular biology and cell culture. Primary human foreskin fibroblasts obtained from the Cell Culture Core Facility of the Yale University Skin Disease Research Center were cultured as described[24] in Dulbecco's modified eagle's medium (DMEM) containing 10% fetal calf serum. Amphotropic retroviruses and shRNA-containing lentiviruses were produced by transient transfection of 293T cells with a transfer vector and amphotropic packaging plasmids encoding VSV-G and gag-pol using Lipofectamine 2000 (Invitrogen). Human cells at 50% confluency were infected for 8–12 h with viral supernatants containing 7 $\mu g\,ml^{-1}$ polybrene. Typically 70–90% infection efficiency was achieved as assessed by a green fluorescent protein (GFP)-encoding viral gene or by immunostaining with haemagglutinin (HA) antibody (Covance). After infection, fresh medium was added to the infected fibroblasts. Some cells were analysed by western blotting for p53 (DO-1, Neomarkers) and β-actin (Abcam). Other cells were fixed with 0.2% glutaraldehyde and stained for β-galactosidase activity at acidic pH according to Dimri *et al.*[25].

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

Received 8 March 2010; accepted 4 February 2011.
Published online 10 April 2011.

1. Mendez, R. *et al.* Phosphorylation of CPE binding factor by Eg2 regulates translation of c-mos mRNA. *Nature* **404,** 302–307 (2000).
2. Tay, J. & Richter, J. D. Germ cell differentiation and synaptonemal complex formation are disrupted in CPEB knockout mice. *Dev. Cell* **1,** 201–213 (2001).
3. Alarcon, J. M. *et al.* Selective modulation of some forms of schaffer collateral-CA1 synaptic plasticity in mice with a disruption of the CPEB-1 gene. *Learn. Mem.* **11,** 318–327 (2004).
4. Wu, L. *et al.* CPEB-mediated cytoplasmic polyadenylation and the regulation of experience-dependent translation of alpha-CaMKII mRNA at synapses. *Neuron* **21,** 1129–1139 (1998).
5. Zearfoss, N. R., Alarcon, J. M., Trifilieff, P., Kandel, E. & Richter, J. D. A molecular circuit composed of CPEB-1 and c-Jun controls growth hormone-mediated synaptic plasticity in the mouse hippocampus. *J. Neurosci.* **28,** 8502–8509 (2008).
6. Burns, D. M. & Richter, J. D. CPEB regulation of human cellular senescence, energy metabolism, and p53 mRNA translation. *Genes Dev.* **22,** 3449–3460 (2008).
7. Groisman, I. *et al.* Control of cellular senescence by CPEB. *Genes Dev.* **20,** 2701–2712 (2006).
8. Campisi, J. & d'Adda di Fagagna, F. Cellular senescence: when bad things happen to good cells. *Nature Rev. Mol. Cell Biol.* **8,** 729–740 (2007).

9. Stewart, S. A. & Weinberg, R. A. Telomeres: cancer to human aging. *Annu. Rev. Cell Dev. Biol.* **22,** 531–557 (2006).
10. Barnard, D. C., Ryan, K., Manley, J. L. & Richter, J. D. Symplekin and xGLD-2 are required for CPEB-mediated cytoplasmic polyadenylation. *Cell* **119,** 641–651 (2004).
11. Kim, J. H. & Richter, J. D. Opposing polymerase-deadenylase activities regulate cytoplasmic polyadenylation. *Mol. Cell* **24,** 173–183 (2006).
12. Kim, J. H. & Richter, J. D. RINGO/cdk1 and CPEB mediate poly(A) tail stabilization and translational regulation by ePAB. *Genes Dev.* **21,** 2571–2579 (2007).
13. Lagos-Quintana, M. *et al.* Identification of tissue-specific microRNAs from mouse. *Curr. Biol.* **12,** 735–739 (2002).
14. Holst, L. M., Kaczkowski, B. & Gniadecki, R. Reproducible pattern of microRNA in normal human skin. *Exp. Dermatol.* **19,** e201–e205 (2010).
15. Liao, J. Y. *et al.* Deep sequencing of human nuclear and cytoplasmic small RNAs reveals an unexpectedly complex subcellular distribution of miRNAs and tRNA 3′ trailers. *PLoS ONE* **5,** e10563 (2010).
16. Katoh, T. *et al.* Selective stabilization of mammalian microRNAs by 3′ adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. *Genes Dev.* **23,** 433–438 (2009).
17. Schmid, M., Kuchler, B. & Eckmann, C. R. Two conserved regulatory cytoplasmic poly(A) polymerases, GLD-4 and GLD-2, regulate meiotic progression in *C. elegans*. *Genes Dev.* **23,** 824–836 (2009).
18. Mullen, T. E. & Marzluff, W. F. Degradation of histone mRNA requires oligouridylation followed by decapping and simultaneous degradation of the mRNA both 5′ to 3′ and 3′ to 5′. *Genes Dev.* **22,** 50–65 (2008).
19. Santos, S. D. & Ferrell, J. E. Systems biology: on the cell cycle and its switches. *Nature* **454,** 288–289 (2008).
20. Ofir-Rosenfeld, Y., Boggs, K., Michael, D., Kastan, M. B. & Oren, M. Mdm2 regulates p53 mRNA translation through inhibitory interactions with ribosomal protein L26. *Mol. Cell* **32,** 180–189 (2008).
21. Takagi, M., Absalon, M. J., McLure, K. G. & Kastan, M. B. Regulation of p53 translation and induction after DNA damage by ribosomal protein L26 and nucleolin. *Cell* **123,** 49–63 (2005).
22. Kwak, J. E., Wang, L., Ballantyne, S., Kimble, J. & Wickens, M. Mammalian GLD-2 homologs are poly(A) polymerases. *Proc. Natl Acad. Sci. USA* **101,** 4407–4412 (2004).
23. Schulte, J. H. *et al.* MicroRNAs in the pathogenesis of neuroblastoma. *Cancer Lett.* **274,** 10–15 (2009).
24. Rangarajan, A., Hong, S. J., Gifford, A. & Weinberg, R. A. Species- and cell type-specific requirements for cellular transformation. *Cancer Cell* **6,** 171–183 (2004).
25. Dimri, G. P. *et al.* A biomarker that identifies senescent human cells in culture and in aging skin *in vivo*. *Proc. Natl Acad. Sci. USA* **92,** 9363–9367 (1995).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank V. Ambros, J. Steitz, T. Kowalik and R. Davis for comments. S.N. was supported by a fellowship of the Max Planck Society and by European Molecular Biology Organization fellowship ALTF 995-2004. This work was supported by National Institutes of Health grant AG30323. Additional core support from the Diabetes Endocrinology Research Center (DK32520) is acknowledged.

Author Contributions Experiments were performed by D.B. and A.D. S.N. designed and constructed luciferase reporter constructs. D.B. and J.D.R. designed the experiments and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of this article at www.nature.com/nature. Correspondence and requests for materials should be addressed to J.D.R. (joel.richter@umassmed.edu).

METHODS

Analysis of p53, Gld2 and Gld4. Lentiviruses expressing shRNAs for Gld2, Gld4 and mitoPAP were generated as described[6]. shRNA against the TET repressor has also been described[6]. A retrovirus expressing Gld2–HA was generated as described[7]. Control and shCPEB infected fibroblasts were cultured in methionine and cysteine-free media (Invitrogen) for 45 min and then cultured in media containing 140 mCi [^{35}S]methionine and [^{35}S]cysteine (ProMix, Amersham) for 30 min. The cells were then washed and cultured in fresh DMEM supplemented with 2 mM each of methionine and cysteine for the times indicated. The cells were then frozen and stored until they were used for p53 immunoprecipitation and analysis by SDS–polyacrylamide gel electrophoresis and phosphorimaging. Cells were also cultured in methionine/cysteine-free media in the presence of MG132, a proteasome inhibitor, for 30 min followed by culture for 15 min in 100 μCi [^{35}S]methionine and cysteine; p53 was then immunoprecipitated and analysed as noted above.

Ligation-mediated polyadenylation test assays were used to detect the poly(A) tail of *p53* mRNA in wild-type cells, shGld2 knockdown cells (two shRNAs targeting different regions of Gld2 were used), cells expressing ectopic CPEB and cells expressing ectopic that lacked a zinc finger and hence unable to bind RNA (CPEBΔZF)[6]. Quantitative RT–PCR analyses were normalized against *actin* RNA.

Oxygen consumption and cellular senescence. To measure oxygen consumption, approximately 4×10^5 cells were washed and suspended in 200 ml Krebs–Ringer solution plus HEPES (125 mM NaCl, 1.4 mM KCl, 20 mM HEPES, pH 7.4, 5 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 1 mM $CaCl_2$) containing 1% BSA. Cells from each condition were aliquoted into a BD Oxygen Biosensor System plate (BD Biosciences) in triplicate. Plates were assayed on a SAFIRE multimode microplate spectrophotometer (Tecan) at 1 min intervals for 60 min at an excitation wavelength of 485 nm and an emission wavelength of 630 nm.

Mock or shGld2-infected cells were stained for β-galactosidase at acidic pH, which denotes cellular senescence. Cell number was also determined with a haemocytometer; population doublings were plotted as growth curves of wild-type cells or cells infected with shGld2.

miR-122 cloning and sequencing. Small RNAs from human foreskin fibroblasts were extracted with mirVANA PARIS kit (Ambion). Those corresponding in length to18–24 nucleotides were resolved by urea–polyacrylamide gel electrophoresis, extracted and ethanol precipitated. miRNA cloning linker-1 (IDT) was ligated to the 3′ ends and used to prime a reverse transcription reaction with Superscript III (Invitrogen) and the RT primer DP3 (5′-ATTGATGGTGCCTACAG-3′). cDNA was then PCR amplified with *miR-122* specific primer 5′-AGGGGCGCCTG GAGTGTGACAATG-3′ and DP3. The PCR product was cloned into pGEM-T (Promega) and sequenced. The chromatogram shown is adapted from 4peaks (http://mekentosj.com/science/4peaks/).

Antagomir depletion of *miR-122*. LNA antagomir against *miR-122*, or a scrambled sequence LNA (Exiqon) were electroporated (Amaxa, Lonza) at a final concentration of 4 nM into approximately 10^6 human foreskin fibroblast cells together with 0.8 μg firefly (*pGL3*, Promega) and 1.0 μg *Renilla* (*pRLTK*) luciferase-encoding plasmid, the latter harbouring the full-length CPEB 3′ UTR or deletion mutations of the 3′ UTR. Luciferase assays were performed 16–24 h after electroporation according to methods described elsewhere[26]. The amount of *Renilla* luciferase activity derived from RNA containing the entire CPEB 3′ UTR (full) was arbitrarily set at 100. When used, the CPEB 3′ UTR deletions (Δ) were (in CPEB nucleotide number) as follows: ΔA, 420–755; ΔB, 480–755; ΔC, 530–755; ΔD, 565–755; ΔE, 640–755.

CPEB-Gld4 co-immunoprecipitation. Cells were transfected with plasmids encoding Flag–CPEB and HA–Gld4 using Effectene (Qiagen). The cells were then collected in PBS and lysed in lysis/wash buffer (50 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM $MgCl_2$, 0.1 mM CCl_2, 0.1% SDS and Complete Protease Inhibitor (Roche)). Extracts (0.5 mg protein) were incubated with M2–Flag antibody-(Sigma) coated Dynabeads (Invitrogen) for 2 h at 4 °C. The beads were then washed three times with lysis/wash buffer and the bound proteins eluted by boiling in SDS sample buffer. Co-immunoprecipitates were detected by western blotting with HA antibody (HA.11 16B12, Covance). Control immunoprecipitations were performed with generic mouse IgG-coated Dyanbeads.

Gld4-RNP co-immunoprecipitation. Mock or CPEB-depleted human foreskin fibroblasts[6] were electroporated (Amaxa, Lonza) with a plasmid encoding Flag–Gld4 according to the manufacturer's instructions. Immunoprecipitation with the Flag antibody followed the procedure of Peritz *et al.*[27] with the following modifications: (1) M2 anti-Flag (Sigma)-coated Dynabeads were used instead of agarose beads; and (2) washes with buffer containing 1 M urea were omitted. *p53* RNA was detected by RT–PCR as described[6].

26. Nottrott, S., Simard, M. J. & Richter, J. D. Human let-7a miRNA blocks protein production on actively translating polyribosomes. *Nature Struct. Mol. Biol.* **13,** 1108–1114 (2006).
27. Peritz, T. *et al.* Immunoprecipitation of mRNA-protein complexes. *Nature Protocols* **1,** 577–580 (2006).